Indo-1 binding to protein in permeabilized ventricular myocytes alters its spectral and Ca binding properties

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ABSTRACT We have examined the binding of the fluorescent Ca indicator indo-1 to cellular protein in permeabilized ventricular myocytes and also to soluble and particulate myocyte protein. Using either a filtration technique or equilibrium dialysis, and conditions similar to those in a cardiac myocyte patch clamped with 100 μM indo-1 in the patch pipette, we found that 72% of the total indo-1 was bound to myocyte protein at a protein concentration of 100 mg/ml. This corresponds to a binding of 3.8 ± 0.5 nmol indo-1/mg protein. Separation of the myocyte protein into a soluble and a particulate fraction showed that 63% of the bound indo-1 was bound to soluble protein, corresponding to a binding of 3.22 ± 0.99 nmol/mg, whereas 37% of the bound indo-1 was bound to particulate protein (0.85 ± 0.14 nmol/mg) at a low [Ca] (pCa ~ 9). Binding of indo-1 in permeabilized myocytes was ~60% higher at a saturating Ca concentration (pCa = 3), than under Ca free conditions (1 mM EGTA). Simultaneous measurements of free [Ca] with a Ca selective electrode and indo-1 fluorescence showed that, the dissociation constant (K_d) for Ca was increased 4–5 fold in the presence of permeabilized myocytes as compared to the value obtained in vitro. In agreement with the binding experiments we estimate that the true K_d and the apparent K_d (using ratiometric measurements) for Ca binding to indo-1 are increased approximately four fold, at a myocyte protein concentration of 100 mg/ml.

INTRODUCTION

The Ca indicator indo-1 is widely used to estimate the cytoplasmic free [Ca] in various cell types. Attempts to make in situ calibrations of fluorescent dyes (Williams et al., 1985; Wier et al., 1987; Borzak et al., 1990; Williams and Fay, 1990), have been hampered by the difficulty of controlling intracellular free [Ca]. The estimation of free [Ca] is generally based on the assumption that the Ca binding and fluorescence characteristics of the indicator are the same in the cytoplasm as in calibration solutions. However, several factors may alter the fluorescence characteristics of indo-1 in cells and thus, affect the quantitation of the free [Ca].

First, a number of fluorescent and metallochromic Ca indicators have been found to bind to cellular proteins in skeletal muscle (Baylor et al., 1982, 1986; Beeler et al., 1980; Maylie et al., 1987*a–c;* Konishi et al., 1988; Hirota et al., 1989). In cardiac myocytes loaded with the acetoxymethylester of the Ca indicators fura-2 and indo-1, it has recently been reported that as much as 68 and 84% of the diffusible dye is bound to cell components (Blatter and Wier, 1990). This would imply that if the cytoplasm is equilibrated with a fixed free [indo-1] (e.g., in a dialyzing patch pipette), the total cytoplasmic [indo-1] may exceed that in the pipette by several fold, and thereby increase the total cellular Ca buffering capacity of the Ca indicator significantly.

Second, indicator properties such as the dissociation constant for Ca (K_d), as well as association and dissociation rates for complexation of Ca by fura-2, are altered in the myoplasm in skeletal muscle, or in the presence of cellular proteins (Baylor and Hollingworth, 1988; Konishi et al., 1988; Uto et al., 1991).

Furthermore, the use of the cell permeable indo-1/AM, is additionally complicated by partial cleavage of the ester form and sequestration in cellular compartments such as mitochondria (Spurgeon et al., 1990). And finally, indo-1 fluorescence from endothelial cells may also interfere with measurements of Ca transients in multicellular preparations during contraction (Lorell et al., 1990).

The aim of the present study is to quantify the binding of indo-1 to components in cardiac myocytes, and examine the possible effects of such binding on spectral characteristics and Ca affinity of indo-1. Two independent methods were used to quantitate the binding of indo-1: (*a*) filtration of digitonin permeabilized cells and cell fractions equilibrated with indo-1; and (*b*) equilibrium dialysis of permeabilized cells. Alteration of the K_d for Ca in the presence of permeabilized myocytes or cellular protein, was examined by simultaneous measurements of free [Ca] with a Ca selective minielectrode and fluorescence emission.

METHODS

The primary preparation used in this study was digitonin permeabilized rabbit ventricular myocytes. However, to examine the effects of cell proteins on indo-1 characteristics more thoroughly, two other preparations were also used. (*a*) Homogenized myocytes were used to allow separation of soluble and particulate protein fractions; and (*b*) intact cardiac myocytes were loaded with indo-1/AM to compare spectral characteristics of indo-1 from these cells to the characteristics of the potassium salt incubated with permeabilized cells.

Myocyte preparation

Rabbit ventricular myocytes were isolated as described by Hryshko et al. (1989). Briefly, New Zealand white rabbit hearts were perfused with

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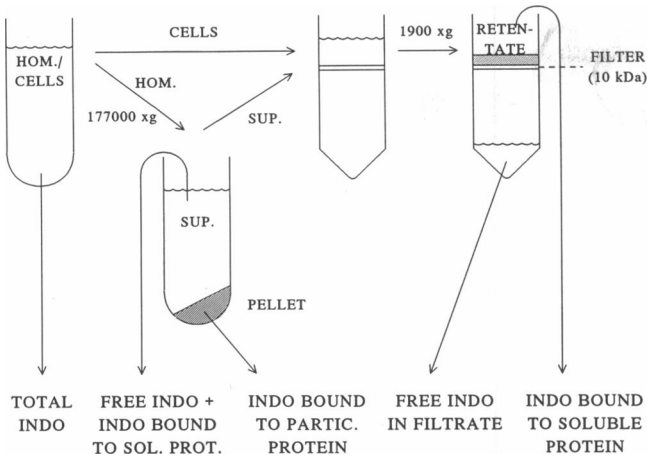

FIGURE 1 Procedure for measurement of indo-1 binding to cellular protein. Binding was measured from the fluorescence obtained by diluting each fraction 100 fold in a KCl buffer with 1 mM EGTA, pH = 7.2 and converting the fluorescence at 470 nm to [indo-1] by a indo-1 standard concentration curve. In homogenized myocytes equilibrated with indo-1, fluorescence was measured before centrifugation at 177,000 × *g*. After centrifugation, fluorescence was measured in the supernatant and the resuspended pellet. Then, the supernatant was spun through a 10 kDa cutoff filter at 1,900 × *g*, and fluorescence was measured in the resuspended soluble protein left on the filter, and the filtrate. Indo-1 bound to particulate protein was obtained as the difference between total indo-1 and indo-1 in the supernatant, whereas indo-1 bound to soluble protein was obtained as the difference between indo-1 in the supernatant and indo-1 in the filtrate. For permeabilized myocytes, fluorescence was measured in the cells equilibrated with indo-1 before filtration, then the cells were spun through a 10 kDa cutoff filter at 1,900 × *g*, and fluorescence was measured in the resuspended cells left on the filter and in the filtrate. Indo-1 bound to cells was measured as the difference between total indo-1 and indo-1 in the filtrate.

a nominally Ca-free solution for 5 min, followed by a 30 min perfusion with 1 mg/ml collagenase B (Boehringer Mannheim Corp., Indianapolis IN) and 0.16 mg/ml protease (type XIV, Sigma Chemical Co., St. Louis, MO). After enzymatic digestion, the heart tissue was teased apart on a plate and then filtered through a nylon mesh. The myocytes were then washed three times in a KCl buffer containing (in mM): KCl 140, NaCl 10, HEPES 10, pH = 7.2 at 23°C. The first wash included 0.5 mM EGTA to lower the free [Ca].

When using permeabilized cells, the isolated cells were incubated with 10 μM digitonin and incubated with indo-1 (from Molecular Probes Inc., Eugene, OR) for 45 min after the final wash in the isolation procedure described above. When using homogenized cells, the cells were incubated with indo-1 for the same period after homogenization. Myocytes loaded with indo-1/AM were obtained by incubating 0.5 ml of concentrated unwashed myocytes for 15 min with 10 μM indo-1/AM (from a 100 μM stock in 90% fetal calf serum, 10% DMSO, 0.006% Pleuronic [F-127]). After this loading, myocytes were washed three times as described above for myocytes used for permeabilization.

Determination of K_d and B_{max} for indo-1 binding to protein

The experimental approach used to evaluate indo-1 binding to cellular proteins used both homogenized myocytes and permeabilized myocytes (see Fig. 1).

Homogenized myocytes. Homogenized myocytes (400 μl, final protein concentration 10 ± 1 mg/ml) were incubated with different concentrations of total indo-1 for 45 min at a free [Ca] below 200 nM. After incubation, the homogenized cells were separated into a particulate and a soluble protein fraction by 30 min centrifugation at 177,000 × *g* (see Fig. 1). The surface of the pellet was gently washed twice to remove residual supernatant, and then resuspended in 200 μl KCl buffer. A fraction of the supernatant (300 μl) was centrifuged through a 10 kDa cutoff filter (Centricon 10 microconcentrator, Amicon, Danvers, MA) at 1,900 × *g* for 45–60 min. The retentate was resuspended by washing with two volumes of 150 μl KCl buffer and vigorous vortexing several times during a 60 min period, and then spun off the inverted filter at 1,900 × *g* for 45 min. Finally, the inverted filter was washed again with 400 μl KCl buffer.

Quantification of indo-1 binding. An aliquot (20 μl) of each fraction, was diluted 100 times in KCl buffer with 1 mM EGTA. The 100-fold dilution of the sample eliminated any significant reduction in fluorescence emission by protein, and assured that all indo-1 was in its Ca free form. Fluorescence emission was measured at the peak of the Ca free indo-1 spectrum (480 nm for Perkin Elmer FP66, and 470 nm for PTI Deltascan). The excitation wavelength was set at the "isosbestic point" for the excitation spectra for indo-1 (355 nm). The indo-1 fluorescence in each fraction was converted to indo-1 concentration using a standard curve, which was obtained by adding known amounts of indo-1 to a KCl buffer with 1 mM EGTA and measure emission at the Ca free peak of the indo-1 spectrum.

In all experiments, the weight and protein concentration (Lowry et al., 1951) was determined for each fraction. The pellet accounted for 4.3 ± 0.4% and the supernatant 93 ± 5% of the initial weight of the homogenate. Thus, 97 ± 1% of the total weight was recovered after the first separation. However, 75% of the total protein was recovered as particulate protein and 25% as soluble protein. 93% of the supernatant volume was recovered after filtration, with 80 ± 5% of the soluble protein in the retentate fraction.

By determining the fluorescence and the [protein] in each fraction, the amount of indo-1 bound to soluble and particulate protein could be quantified in nmol/mg protein. The corresponding free [indo-1] was taken as that in the filtrate. The amount of indo-1 bound to particulate protein was determined as indo-1 recovered in the resuspended pellet fraction. Indo-1 bound to soluble protein was measured as indo-1 recovered in the resuspended retentate + indo-1 washed of the filter after resuspension of retentate.

The indo-1 bound to soluble protein was also determined as the difference between indo-1 content in the supernatant and the filtrate fractions. Similarly, the indo-1 bound to particulate protein was determined as the difference between the indo-1 content in the homogenate and the supernatant fractions. The latter approach allows a quantitation of indo-1 bound to soluble and particulate protein that does not depend on the recovery of protein after centrifugation and filtration. Both methods of calculating indo-1 binding gave similar results.

Control experiments were carried out with ^{14}C-sucrose, which is assumed not to bind to protein. Samples from each fraction were diluted into 6 ml scintillation cocktail (Beckmann protein +). The ^{14}C-sucrose passively trapped in the pellet and the retentate was used to assess the trapped volume and was expressed as a percentage of the ^{14}C-sucrose in the homogenate. We found 2.4% of the indo-1 content trapped in the pellet fraction and 8.4% in the retentate fraction. These values were subtracted from the indo-1 fluorescence in the respective fractions.

Binding of indo-1 to cellular protein at different pCa was estimated by incubating 400 μl of homogenized myocytes (homogenate [protein] = 9.9 ± 0.7 mg/ml) with 200 μM total indo-1 in the presence of 1 mM EGTA or 1 mM Ca for 45 min. Separation of particulate and soluble protein was carried out as described above. The binding of indo-1 to cellular protein fractions in the presence of 1 mM Ca was normalized to the corresponding binding in the presence of 1 mM EGTA for each experiment.

Permeabilized myocytes. Indo-1 binding experiments were carried out as described for homogenized myocytes, except that no centrifuga-

tion step was performed (Fig. 1). The permeabilized myocytes were incubated at different protein concentrations (5–89 mg/ml) and total indo-1 concentrations (125–450 μM). The cells with a free [indo-1] of 109 ± 7 μM (measured as the [indo-1] in the filtrate) and 10 μM digitonin, were put directly on the 10 kDa cutoff filter and spun at 1,900 × g for 4 h before the retentate was redissolved and fluorescence measured in each fraction.

Equilibrium dialysis. Binding to permeabilized myocytes was also quantified with a modified procedure for equilibrium dialysis described by Beeler et al. (1980). Briefly, 250 μl cells was dialyzed against 1,200 μl of buffer, using Semi-Micro Tubing, MW cut-off 12,000–14,000 (Spectrum Medical Industries, Los Angeles, CA). Fluorescence emission was measured from the cell suspension in the dialysis bag and the bulk solution before and after 48 hours of dialysis at 4°C. The emission wavelength was set at 470 nm and samples were diluted 100 fold in KCl buffer with 1 mM EGTA as described for the filtration experiments. The average free [indo-1], as measured in the buffer solution after dialysis was 107 ± 5 μM. For simplicity, the binding of indo-1 to protein was assumed to follow Michaelis-Menten kinetics [$B = B_{max} \times X/(K_d + X)$], and data were fitted by a least squares procedure, to obtain the K_d and B_{max} values.

Calcium selective minielectrodes

To obtain the dissociation constant for Ca binding to indo-1, fluorescence emission scans for indo-1 were carried out at different free [Ca] in the presence or absence of protein. The free [Ca], which is crucial in the determination of the K_d, was monitored with a Ca selective minielectrode.

The minielectrodes were prepared as described by Schefer et al. (1986) from the following components (all from Fluka Chemical Corp, Ronkonkoma, NY): ETH-129 25 mg, *N*-phenyl-octyl-ether 451.5 μl, tetraphenylborate 8.9 mg, Polyvinylchloride 250 mg. The ETH-129 was dissolved in *N*-phenyl-octyl-ether by vigorous stirring for 15–30 min. Polyvinylchloride was dissolved in ~5 ml tetrahydrofuran and tetraphenylborate was added. Then, the dissolved ETH-129 was added to the dissolved polyvinylchloride.

A batch of Ca selective minielectrodes were then prepared by dipping polyethylene tubes (Clay Adams PE-280, I.D. = 2.15 mm) in the ETH 129 membrane solution. Tetrahydrofuran was allowed to evaporate from the electrode membrane overnight, and the electrodes were then stored until use. Three days before use, the electrode was filled with a solution containing (in mM) KCl 140, NaCl 10, HEPES 10, EGTA 1, and $CaCl_2$ 0.986, to give a pCa ~ 5 at pH = 7.2. Electrode calibration solutions were prepared as described by Bers (1982). Electrodes had a resistance of 1–5 MΩ. In the absence of protein, electrodes showed a Nernstian response (28.5 ± 0.3 mV/decade) down to a free [Ca] of 30 nM or less for more than two weeks. The electrodes drifted less than 0.1 mV/h and the response was 95% complete in less than 1 min above 30 nM free [Ca].

Homogenized or permeabilized myocytes (more than 5 mg/ml protein), shifted the electrode response by 1–8 mV at the first protein exposure, but did not show any significant offsets on subsequent exposures. Therefore electrodes were always preexposed to protein to avoid any offset during the Ca titration. Above 30 nM free [Ca] the Nernstian slope was not altered significantly by exposure to protein, but the response time was slowed significantly below 300 nM Ca (1–30 min for 95% complete response) depending on the protein concentration. In general, the electrodes were affected more by protein from homogenized myocytes than by permeabilized myocytes. Fig. 2 shows the electrode response of an electrode preexposed to protein, before and after a Ca-titration of permeabilized cells incubated with indo-1 (30 mg/ml protein), and one day after the Ca titration. Electrodes were normally discarded after a titration experiment.

Emission spectra and determination of K_d for Ca of indo-1

Fluorescence emission spectra. Fluorescence emission spectra were obtained for the pentapotassium salt of indo-1 in vitro as well as for the salt incubated with permeabilized myocytes and myocytes loaded with indo-1/AM.

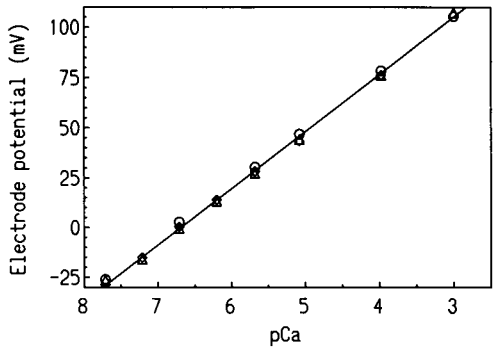

FIGURE 2 Electrode calibration curves. Electrode response is shown after preincubation with cells (*circles*), after Ca titration of cells incubated with indo-1 (*diamonds*) and one day after a Ca titration (*triangles*). The electrodes showed no significant changes in slope (between pCa 7.5 and pCa 3) after preincubation with cells. Only the response time was slowed significantly by this intervention.

Indo-1/AM loaded myocytes were diluted to a final concentration of ~1 mg/ml protein and incubated with 1 mM EGTA and 1 μM of the Ca ionophore 4-Bromo A-23187 for 15 min before a fluorescence emission scan was run (380–520 nm) with 355 nm excitation wavelength. Then 2 mM $CaCl_2$ was added and the emission scan was repeated.

For permeabilized myocytes, the cells were incubated with 10 μM digitonin and 1–5 mM EGTA for 1 h in a total volume of 3 ml. pH was adjusted to 7.2. Autofluorescence was measured before addition of 15 μM indo-1. Free [Ca] was monitored with a Ca selective minielectrode which had been preincubated with permeabilized myocytes, and pCa was increased in steps of 0.3–0.6 pCa units from pCa > 7.5 to pCa < 4. Fluorescence emission scans (380–520 nm) with 355 nm excitation were done after each Ca addition.

Dissociation constant K_d. The dissociation constant K_d for Ca binding to indo-1 is related to fluorescence emission and [Ca] by the equation (Grynkiewicz et al., 1985): $[Ca] = K_d \times [(R-R_{min})/(R_{max}-R)] \times (S_{f2}/S_{b2})$, where R is the ratio of fluorescence at the two wavelengths λ_1 and λ_2. R_{min} and R_{max} represent the values of R for the Ca-free and Ca-saturated indo-1, respectively. S_{f2} is the fluorescence intensity at λ_2 in the absence of Ca and S_{b2} the fluorescence intensity at λ_2 in the presence of a saturating [Ca]. The apparent dissociation constant is $K_d^* = K_d \times (S_{f2}/S_{b2})$. Thus, the K_d^* was determined by measuring the fluorescence emission at λ_1 = 390 nm and λ_2 = 470 nm, and varying the free [Ca] from less than 1 nM to 1 mM. Similarly, the K_d was determined using the fluorescence at a Ca sensitive wavelength (λ_1 = 470 nm) divided by the fluorescence at a Ca independent or isosbestic wavelength, (λ_2 = IB), where $S_{f2}/S_{b2} = 1$. The K_d values were determined in KCl buffer, in the presence of permeabilized myocytes as well as soluble and particulate protein.

The fluorescence emission ratios were plotted as a function of pCa. Data were fitted by a sigmoid plot to obtain the K_d and K_d^* as well as R_{max} and R_{min}. The Hill coefficient of the sigmoid plot was compared to the ideal Hill coefficient of 1 for 1:1 Ca to indo-1 binding.

RESULTS

Indo-1 emission spectra

Fig. 3 shows the emission spectra of Indo-1 at free [Ca] from less than 1 nM to 100 μM. The spectra in KCl buffer (in vitro) exhibits the characteristic emission

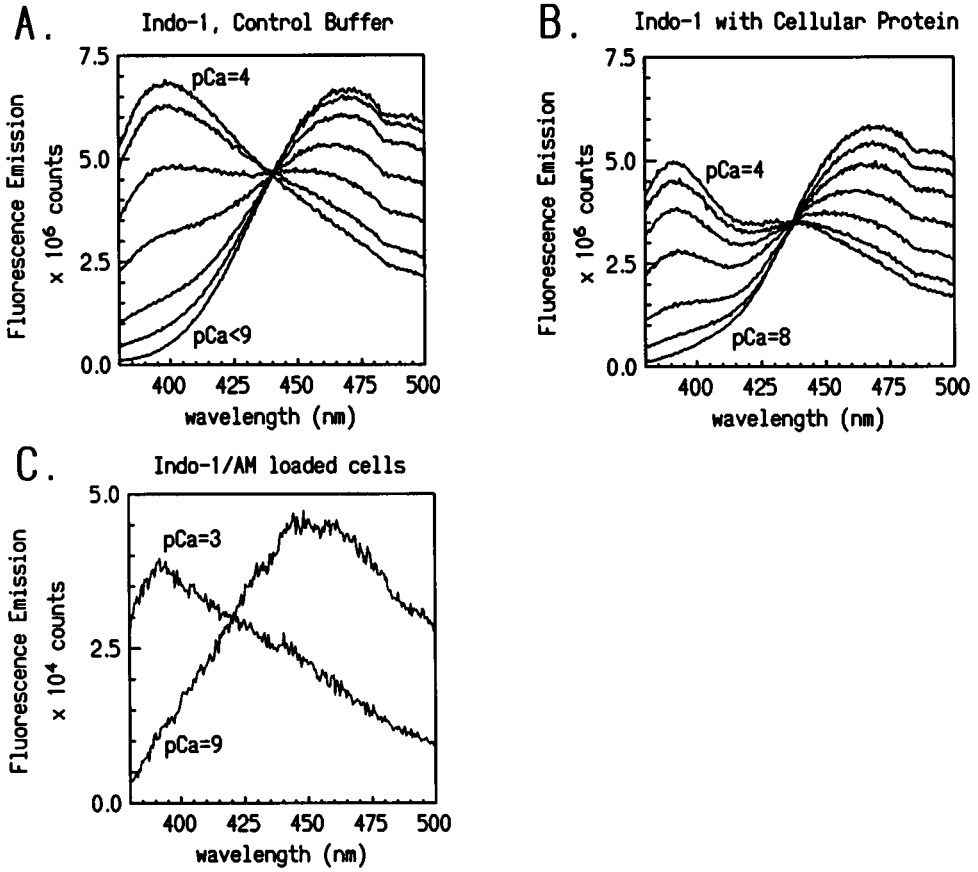

FIGURE 3 Dependence of indo-1 emission spectra on experimental conditions. (*A*) Indo-1 fluorescence emission at increasing free [Ca]. Emission scans were obtained in a KCl buffer containing 140 mM KCl, 10 mM NaCl, 10 mM Hepes, 10 μM digitonin, 10 μM indo-1, 1.0 mM EGTA, pH = 7.2, and 0–1 mM Ca. (*B*) Myocytes with 10 μM indo-1 and 1 mM EGTA in KCl buffer (3 mg/ml protein) were permeabilized with 10 μM digitonin and titrated with Ca from 10 nM to 100 μM. (*C*) Myocytes were loaded with indo-1/AM before dilution into a KCl buffer with 1 mM EGTA and 1 μM Br-A23187. Emission scans were recorded before and after addition of 2 mM total Ca.

peaks at 400 nm for the Ca bound and at 470 nm for the Ca free indicator (Fig. 3 *A*). The isosbestic point, where fluorescence emission is independent of Ca was 443 ± 1 nm in vitro. Fig. 3 *B* illustrates the changes in the emission characteristics for indo-1, when the pentapotassium salt of indo-1 was incubated with permeabilized ventricular myocytes (3 mg/ml). The Ca bound peak showed a blueshift (5–15 nm, depending on protein concentration), and the amplitude of the peak was reduced relative to the Ca free peak. The Ca free peak showed virtually no shift in emission wavelength. Furthermore, the emission spectrum showed an overall reduction of fluorescence emission, and in particular at wavelengths below 450 nm. In agreement with this, the absorption spectrum for permeabilized myocytes showed a broad peak around 410 nm (Fralix et al., 1990).

Fig. 3 *C* shows the emission spectra of indo-1 from ventricular myocytes which were loaded with indo-1/AM. In contrast to cells incubated with the pentapotassium salt, the indo-1/AM loaded cells shows a 20 nm blueshift for the Ca-free peak. The Ca-bound peak also showed a blueshift from 400 to 390 nm. The uncleaved indo-1/AM shows a prominent peak around 450 nm (data not shown), which is only weakly affected by Ca. However, the peak around 450 nm in the indo-1/AM loaded cells, was sensitive to changes in free [Ca], and thus, the blueshift of this peak appears to be related to sequestration, partial cleavage of indo-1/AM, and/or binding of indo-1 intracellularly. These complications in the interpretation of the fluorescence emission spectrum for the AM-loaded cells, prompted us to concentrate on an examination of the binding of the free acid form of indo-1 to cellular protein, and the possible alteration in Ca binding characteristics in the presence of cellular protein.

Indo-1 binding to cellular protein

Fig. 4 illustrates the binding of indo-1 to permeabilized myocytes as a function of total protein. Based on pilot studies the total [indo-1] was adjusted so that the average free [indo-1] was maintained at 109 ± 7 μM. This may simulate conditions often used in patch pipettes. Fig. 5 shows the bound indo-1 (in percent of total indo-1) as a function of total protein from both filtration and equilibrium dialysis experiments. The data were fit by a curve for a single binding site [$B = B_{max} \times X/(K_d + X)$], and it

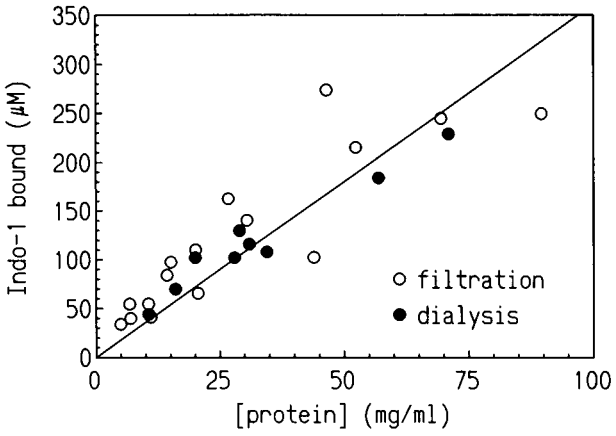

FIGURE 4 Total indo-1 bound to protein. Binding was measured as indo-1 in the cell fraction before filtration minus indo-1 in the filtrate (*open symbols*). Indo-1 was also measured by equilibrium dialysis. Indo-1 in the dialysis bag before dialysis was subtracted from the indo-1 in the bag after dialysis (*filled symbols*). The average free [indo-1] was 108 ± 5 μM.

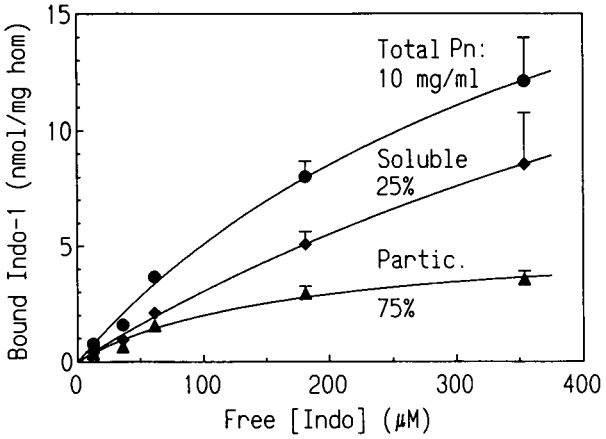

FIGURE 6 Binding of indo-1 to particulate and soluble protein. Binding was measured by centrifugation and filtration of 10 mg/ml homogenate protein equilibrated with different indo-1 concentrations. K_d for indo-1 binding was 433 μM for total, 912 μM for soluble, and 171 μM for particulate protein. The corresponding B_{max} values were 27.0, 30.7, and 5.4 nmol/mg homogenate respectively.

appeared that at cellular protein concentrations (i.e., ~100 mg/ml, more than 72% of the total indo-1 was bound (50% of indo-1 was protein-bound at 30 mg/ml). This value is similar to that found by Blatter and Wier (1990). The amount of indo-1 bound per mg protein, at 109 ± 7 μM indo-1, averaged 3.8 ± 0.5 nmol/mg (n = 8). In agreement with this, dialysis experiments (free [indo-1] = 107 ± 5 μM) resulted in binding of 3.6 ± 0.2 nmoles/mg cellular protein.

To examine the binding of indo-1 to soluble and particulate protein from cardiac myocytes we used homogenized myocytes. The binding of indo-1 to protein was examined as a function of free indo-1 to estimate the K_d and B_{max} for binding of indo-1 to soluble and particulate protein. Fig. 6 shows the binding of indo-1 to soluble and particulate protein from homogenized isolated ventricular myocytes. The K_d for indo-1 was 433 μM for total, 912 μM for soluble and 171 μM for particulate protein. The corresponding B_{max} values were 27.0, 30.7 and 5.4 nmol/mg homogenate respectively. Thus, at a free indo-1 of 100 μM, soluble protein only accounts for 25% of the total protein, but binds 63% of the total indo-1 bound.

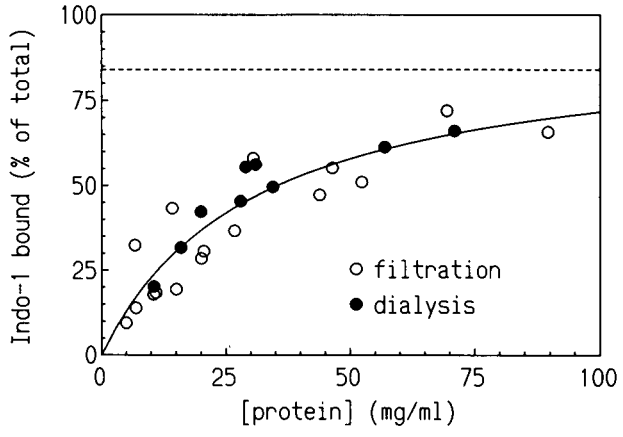

FIGURE 5 Fraction of indo-1 bound to cellular protein. Bound indo-1, measured as described in Fig. 4, was normalized to the total indo-1 concentration and plotted versus total [protein]. The free indo-1 was 108 ± 5 μM. The dotted line represents bound indo-1 estimated, by Blatter and Wier (1990).

Finally, binding of indo-1 to particulate and soluble protein was determined in the presence of 1 mM free [Ca] where indo-1 is saturated with Ca, and with 1 mM EGTA where indo-1 is in its Ca free form. The absolute binding of indo-1 to protein varied between experiments, with an average of 3.22 ± 0.99 nmol bound per mg soluble protein, and 0.85 ± 0.14 nmol bound per mg particulate protein at 100 μM free indo-1 and 1 mM EGTA. Fig. 7 shows the binding of indo-1 to particulate and soluble protein at 1 mM free [Ca], normalized to the binding in the presence of 1 mM EGTA. There was ~60% more indo-1 bound to both soluble and particulate protein when incubated with 1 mM Ca as compared to incubation with 1 mM EGTA. Thus, the Ca bound form of indo-1 has higher affinity for protein binding.

Determination of the K_d of indo-1 for Ca

The K_d of indo-1 for Ca was determined in the absence and presence of particulate and soluble protein as well as permeabilized myocytes. Fig. 8 shows typical calibration curves for indo-1 in the absence and presence of permeabilized myocytes. The K_d in Fig. 8, as determined from the 470 nm/isosbestic fluorescence emission ratio, was increased from 180 nM in vitro to 454 nM in the pres-

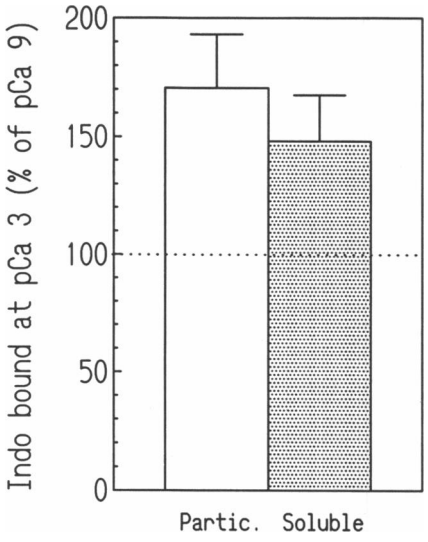

FIGURE 7 Dependence of indo-1 binding on free [Ca]. The binding of indo-1 to particulate and soluble protein was determined at a saturating Ca concentration (1 mM) and in the presence of 1 mM EGTA where indo-1 is in the Ca free form. The indo-1 bound at a free [Ca] of 1 mM was normalized to the binding with 1 mM EGTA (100% equals 0.85 ± 0.14 nmol/mg for particulate protein and 3.22 ± 0.99 nmol/mg for soluble protein, n = 5).

ence of 12 mg/ml protein. Data fitted by a sigmoid curve gave an average K_d of 239 ± 32 nm and K_d^* of 600 ± 49 nm (for λ_1/λ_2 = 390/470) for the in vitro calibration in KCl buffer with 1 mM EGTA and pH = 7.2. The in vitro calibration curve showed Hill coefficients in the range 0.9 to 1.1. This was also true for particulate and soluble protein at the protein concentrations examined, while permeabilized cells showed considerable variation in the Hill coefficient with curves for the 390/470 ratio showing a typical Hill coefficient of 0.4 to 0.6 in the presence of more than 20 mg/ml protein.

Fig. 9 *A* shows K_d values obtained from the 470 nm/isosbestic fluorescence emission ratio at different [protein]. The K_d increased with increasing protein at all [protein] examined. Diamonds represent values obtained in permeabilized cells whereas triangles and circles represent values obtained in the presence of particulate and soluble protein respectively. The K_d^* obtained from the 390/470 nm ratio is shown in Fig. 9 *B* as a function of the protein concentration. The increase in K_d^* with increasing [protein] levelled off at higher protein concentrations. Extrapolation to an intact cellular protein concentration, (assumed to be ~100 mg/ml total protein) gave a K_d^* of 3 μM for indo-1 incubated with permeabilized myocytes.

DISCUSSION

The main findings of the present study are (*a*) Indo-1 emission spectra are altered in the presence of myocyte protein. (*b*) 72% of the total indo-1 is bound to protein in permeabilized myocytes. (*c*) Binding of indo-1 to protein increases the K_d for Ca 4–5 fold.

Fluorescence emission spectra

The in vitro emission spectra for the pentapotassium salt of indo-1, and its Ca dependency found in the present experiments (K_d = 239 ± 32 nM) are similar to values previously reported (Grynkiewicz et al., 1985; Popov et al., 1988). However, the emission spectra for proteins or permeabilized myocytes incubated with indo-1 or loaded with the indo-1/AM show significant differences from the in vitro spectra.

Fluorescence emission is reduced at all wavelengths in the presence of cellular protein. The reduction increases with increasing protein concentration and is more pronounced at wavelengths below 450 nm, which agrees with the absorption spectrum for myocytes which shows a broad peak between 400 and 430 nm (Fralix et al., 1990). However, absorption of fluorescence may not entirely account for the changes in the emission spectra, because permeabilized cells incubated with pentapotassium indo-1 exhibits a blueshift of the emission peak for the Ca-bound indo-1 from 400 to ~390 nm, whereas indo-1/AM loaded cells shows a 20 nm blueshift of the peak at 470 nm. Thus, the spectral changes of indo-1 in the presence of cellular protein indicates that the characteristics of the Ca indicator may be altered by the presence of cellular protein as has been reported for other Ca indicators (Beeler et al., 1980; Konishi et al., 1988; Baylor and Hollingworth, 1988; Uto et al., 1991).

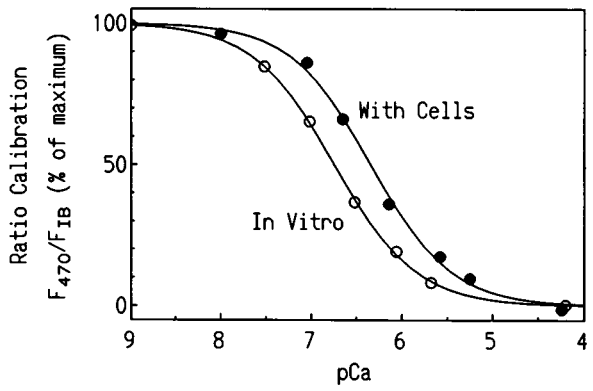

FIGURE 8 Indo-1 calibration curves in vitro and in the presence of 12 mg/ml cellular protein. Permeabilized myocytes were incubated in a KCl buffer and titrated to pCa ~ 3.5 with $CaCl_2$ as described in Fig. 3. Indo-1 fluorescence emission was measured after each Ca addition, and free [Ca] was monitored simultaneously with a Ca selective minielectrode. The K_d for Ca was determined by plotting the fluorescence ratio F_{470}/F_{IB} versus the free [Ca] as measured with the Ca selective electrode. Data were fitted with a sigmoid curve to obtain the K_d values in vitro (*open symbols*), or in the presence of permeabilized myocytes (*filled symbols*).

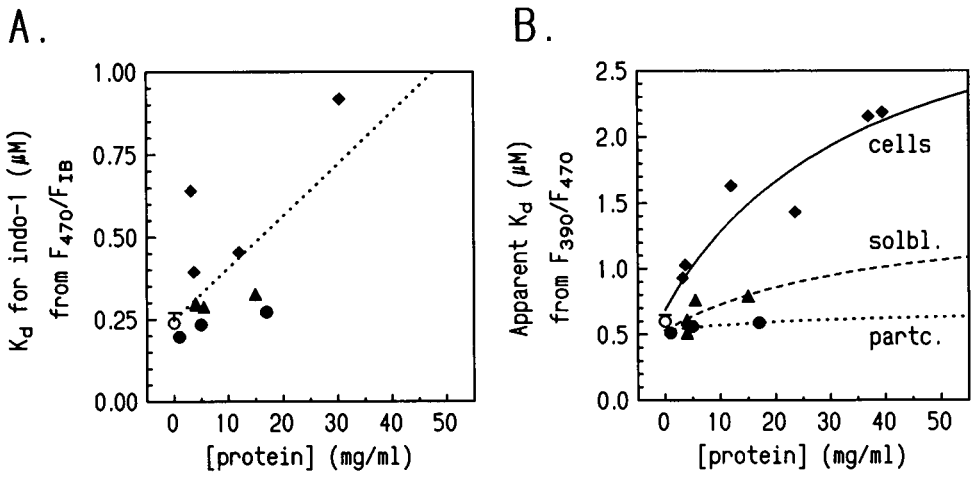

FIGURE 9 (*A*) Change in K_d with increasing [protein]. Values were obtained by plotting the fluorescence ratio F_{470}/F_{1B} versus free [Ca] as described in Fig. 8. (*B*) Change in K_d^* with increasing [protein]. Values were obtained from the fluorescence ratio F_{390}/F_{470}. Results are from in vitro calibrations (*open circle*), permeabilized myocytes (*diamonds*), particulate protein (*circles*) or soluble protein (*triangles*).

Binding of Ca indicators to cellular protein

The present approach, gives a direct quantitation of the indo-1 bound as a function of cellular protein. No direct measurements of the total cellular protein concentration is available for cardiac myocytes, but a value of ~100 mg/ml seems reasonable based on 121 mg homogenate protein/g wet weight × 0.6 mg cell protein/mg homog. protein × 1.06 g wet weight/cm^3 heart × 1 cm^3 heart/0.73 cm^3 cell. Furthermore, a total [protein] of 150 mg/ml would only increase indo-1 binding from 72 to 78% of total indo-1. Binding of indo-1 to the extracellular surface of the myocyte could lead to overestimation of the protein bound indo-1 inside intact cells. However, the sarcolemmal protein only constitutes about 3% of the particulate protein. This, together with the greater indo-1 binding to soluble protein makes it more likely that most of the indo-1 is bound to intracellular proteins.

Both filtration of permeabilized myocytes equilibrated with indo-1 and equilibrium dialysis showed that at a free indo-1 of 107 μM and total [protein] = 100 mg/ml, 72% of the total indo-1 is protein bound. This value is similar to the estimate of 80–85% based on fluorescence recovery after photobleaching by Blatter and Wier (1990).

A value of 100 μM indo-1 was chosen to simulate conditions often used in a patch pipette. Thus, one would expect total indo-1 in the cell to rise to ~4 times that in the pipette with which it is in equilibrium. Fractional centrifugation of homogenized myocytes and filtration of the supernatant showed that 45% of the total indo-1 was bound to soluble proteins, while 27% was bound to particulate protein and 28% was free in the cytosol. Thus, we find that 73% of the total indo-1 is recovered in the cytosolic fraction. This compares well with the results of Lee et al. (1988), who found that in heart tissue loaded with indo-1/AM, 72% of the indo-1 was recovered in the cytosolic fraction after homogenization and centrifugation. Because indo-1 bound to soluble protein amounts to 63% of the total bound indo-1, dialysis of soluble protein out of the myocyte by a patch pipette could be a potentially important factor in determining the total intracellular indo-1. No direct measurements has been done on diffusion of indo-1 and soluble protein during patch clamping. However, the diffusion of protein out of the cell is expected to be much slower than diffusion and binding of indo-1 in the cell.

A four-fold increase in the total intracellular indo-1 (due to binding of indo-1 to cellular protein) would increase the buffering of the Ca transient by indo-1. This, in turn can alter the apparent kinetics of the Ca_i transient. Furthermore, the present results show that binding of indo-1 to cellular protein is ~60% higher for the Ca saturated dye as compared to indo-1 in its Ca free form. The Ca-dependence of indo-1 binding to protein may further complicate the kinetics of the Ca_i transient because the fraction of indo-1 binding to protein may change during a Ca transient (changing the calibration of indo-1).

Recently, it has become possible to measure the intracellular Ca transient and the Ca current across the sarcolemma simultaneously. This makes it possible to quantitate cellular Ca movements during the excitation-contraction coupling (Sipido and Wier, 1991), which is an important step towards a more complete understanding of this process. However, such quantitation relies on assumptions regarding the characteristics of cellular Ca buffers including the intracellular concentration of the Ca indicator. The present data provides a direct measure of the intracellular binding of indo-1 and should together with data by Konishi et al. (1988) and Blatter and Wier (1990) make it possible to improve estimates of the total intracellular fura-2 and indo-1 concentration and Ca buffering capacity.

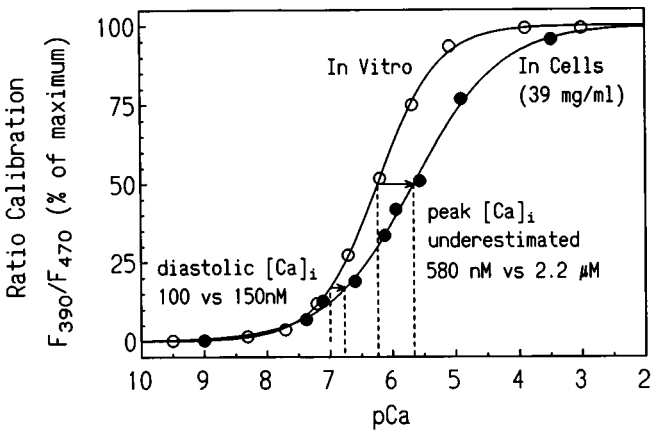

FIGURE 10 Effect of shift in apparent K_d on estimates of free [Ca]. Indo-1 calibration curves were obtained for the fluorescence ratio F_{390}/F_{470} as described for the true K_d in Fig. 8 in the absence (○) or presence of 39 mg/ml myocyte protein (●). If one assumes the in vitro K_d is valid in cardiac myoplasm one might estimate resting $[Ca]_i$ to be 100 nM and peak $[Ca]_i$ to be 580 nM (*tails of arrows*). In the presence of 39 mg/ml cardiac myocyte protein these same fluorescence ratios would correspond to a resting $[Ca]_i$ of 150 nM and a peak $[Ca]_i$ of 2.2 μM (*arrowheads*).

Alterations in indicator K_d for Ca by cellular protein

The present data suggests that both K_d and K_d^* for Ca are changed in the presence of cellular protein. When homogenized myocytes are separated into soluble, and particulate fractions, we find that at a given protein concentration below 20 mg/ml, the shift in K_d is larger in the presence of soluble protein compared to particulate protein (consistent with higher binding of indo-1/mg soluble protein). The K_d is increased 4–5 fold in the presence of permeabilized myocytes, and appears to reach a maximum at higher protein concentrations. However, at protein concentrations above 15 mg/ml, the Hill coefficient for the fluorescence ratio versus [Ca] curve is decreased below 1 (see Fig. 10). This may be explained by a larger shift in K_d at saturating Ca concentrations due to the higher binding of Ca saturated indo-1 to protein (Fig. 7). Thus, at the higher [Ca] the larger protein binding of indo-1 is expected to cause a greater shift in the K_d^*. This would result in an apparent Hill coefficient < 1.

The extrapolated K_d^* at 100 mg/ml cell protein is 4–5 times larger than the in vitro value (Fig. 9 *B*) which is in contrast to the results of Ikenouchi et al. (1991). They reported no significant shift in K_d for indo-1 in cardiac myocytes loaded with indo-1/AM. However, their measurements may suffer from loss of soluble protein during the experiment which is expected to reduce the shift in K_d. On the other hand, our results agree with results obtained using fura-2 where Konishi et al. (1988) and Uto et al. (1991) found that binding of fura-2 to proteins causes a maximal shift in the K_d of ~four fold.

Fig. 10 summarizes the effect of permeabilized myocytes on the relation between free [Ca] and fluorescence ratio. The fluorescence ratio (390/470 nm) is plotted versus the free [Ca] in the absence (*open circles*) and in the presence of 39 mg/ml protein (*filled circles*). Estimates of free [Ca] for a given ratio signal using the in vitro calibration curve differ significantly from values obtained using the calibration curve in the presence of permeabilized myocytes. For example, a fluorescence ratio yielding an apparent diastolic free [Ca] of 100 nM (using the in vitro calibration) would actually be 150 nM in the presence of 39 mg/ml myocyte protein (shown by *lower arrow*). At higher free [Ca] the difference in calibration curves becomes even more critical. Thus, an apparent in vitro peak systolic free [Ca] of 580 nM would be shifted to 2.2 μM in the presence of 39 mg/ml protein (*upper arrow*).

In agreement with this, Hirota et al. (1989) and Konishi and Baylor (1991) found that peak $[Ca]_i$ in cut skeletal fibers measured with PDAA, which only binds weakly to cellular components, was five fold higher than peak $[Ca]_i$ as measured with antipyrylazo III. Thus, at present it seems reasonable to correct the in vitro K_d value for indo-1 for alterations due to binding to cellular proteins. Based on the present data, we suggest that the K_d for cells patched with indo-1 included in the patch pipette should be around 1 μM instead of the value of 250 nM commonly used.

The work was supported by a PhD grant from the University of Århus, Denmark, the Danish Research Academy and a grant from the United States Public Health Service, HL30077.

Received for publication 22 October 1991 and in final form 11 February 1992.

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